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(54) Title: DNA SEQUENCE AND PROTEIN OF THE NON-STRUCTURAL READING FRAME I OF THE HUMAN PARVOVIRUS B19

(57) Abstract

DNA sequences of the human parvovirus B19 genome coding for the non-structural protein, recombinant DNA molecules containing such sequences, antigens coded within these sDNA sequences of the parvovirus B19 genome coding for the non-structural protein sequences, production of those antigens, generation of antibodies or antibody related molecules directed to such antigens, use of these sequences, antigens, antibodies or antibody related molecules for diagnostic, therapeutic or vaccination purposes.

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DNA sequence and protein of the non-structural reading frame I of the human parvovirus B19

DESCRIPTION

Technical field of invention

This invention relates to DNA sequences of the human parvovirus B19 (human parvovirus B19) genome coding for the non-structural protein designated NS-1 and the encoded protein for use in any kind of active or passive vaccination, as a pharmaceutical composition, or as part of a diagnostic composition with special emphasis on the detection of antibodies directed against this protein in infected persons. This facilitates the diagnosis and treatment of a human parvovirus B19 infection with severe complications like e.g. arthritis and aplastic crisis associated with this virus.

It also deals with the production of the protein and subfragments thereof in different procaryotic or eucaryotic systems, especially by the use of recombinant DNA technology.

Furthermore, it relates to the use of this protein for the production of specific antisera, antibodies or antibody related proteins in animals or other appropriate systems and their use for diagnostic or pharmaceutical purposes especially for the detection of the antigen in infected cells.

Background art

The human parvovirus B19 causes a variety of clinically different illnesses in the infected person. The most common picture is the so called *erythema infectiosum* or fifth disease (Anderson et al., 1985). In immunocompromised people or people with need for an elevated erythropoesis, the virus may cause severe aplastic crisis. The outcome of infection in such cases may be lethal. This may also happen if a human parvovirus B19 infection is aquired during pregnancy, since the virus has been shown to be embryopathic by causing fetal hydrops. Infection of adults, mainly women, is often complicated by severe arthritis, that may become chronic and closely resembles rheumatoid arthritis (White et al., 1985; Reid et al., 1985). The life cycle of the virus begins with infection via the nasopharyngeal route (Anderson et al., 1985). Subsequently the virus heavily replicates in the erythropoetic

precursor cells in the bone marrow of the infected individual. This leads to destruction of these cells, the complete shutdown of erythropoesis and high titer viraemia. The virus is thought to be cleared rapidly by the humoral immune response after viraemia followed by the restoration of erythropoesis in the bone marrow. This picture had to be modified for findings in the last years, where the virus was shown to be present in different tissues of the host for prolonged time spans (Saal et al., 1992; Patou et al.; 1992; Foto et al., 1993; Cassinotti et al., 1993).

The diagnosis of infection with human parvovirus B19 is usually based on the detection of antibodies against the structural proteins VP1 and VP2 by ELISA, Western blot or immunofluorescence tests. In some cases the establishment of persistent infection by the virus has been demonstrated by PCR analysis. It has been suggested, that persistent infection may be the cause for the development of the more severe outcome of a human parvovirus B19 infection (Kutzman et al., 1989; Foto et al., 1993; Cassinotti et al., 1993). So far, diagnosis of persistent infection is often based on PCR data, since serological markers for viral persistence do not exist. This widespread routine use of this method is limited due to the necssary operational input by the investigator making other test systems desirable. The availability of such a marker would facilitate the identification of a human parvovirus B19 infection as the cause of one of the above mentioned symptoms and would influence the subsequent therapeutic strategy.

The presence of DNA by itself does not tell anything about the status of the genome with respect to its activity. In contrast the finding of the earliest and key protein in the viral life cycle within a patients cells is indicative for an active virus. This relates to the presence of antibodies against the NS-1 protein as well as to direct demonstration of the protein itself. The production of specific antisera or antibodies against the NS-1 protein allows the detection of the antigen in any kind of cells of the patients. Detection of the NS-1 protein points to an active viral genome in the cell even in the absence of measurable virus titers in the patient sera. This accounts especially for the investigation of bone marrow cells, where the presence of viral DNA has been described, whereas at the same time no virus has been found in the serum by PCR (Foto et al., 1993).

It was one aim of the current invention to develop a new assay for cases in which atypical infection leads to severe clinical complications. In accordance with the present invention it was now surprisingly found that in such cases, in particular in cases with persistent or prolonged parvovirus B19 infection, such infections may be detected by the demonstration of NS-1 specific antibodies in patients' sera. A further object of the invention was to isolate the DNA sequence coding for the parvovirus B19 NS-1 protein. This sequence was used for cloning and expression of the non-structural protein NS-1 in different expression systems. Its suitability for the detection of antibodies in sera from patients with human parvovirus B19 associated complications and suspected persistent infection was demonstrated. Additionally, we have raised antisera against the purified recombinant protein as tools for the search for the antigen in cells from such persons. Furthermore, the protein, antibodies or antibody derivatives are useful in the generation of pharmaceutical compositions for therapeutic or vaccination purposes.

Description of the invention

The current invention relates to the use of a DNA sequence coding for a protein having the biological activities of human parvovirus B19 NS-1 protein and a protein having the biological activities of the non-structural protein NS-1 of the human parvovirus B19 (Fig. 1). The NS-1 protein is the first protein expressed during the course of an infection with the human parvovirus B19 and functions during the replication of the virus. Biological activities ascribed to this protein include a helicase, an ATPase, and a transcriptional regulation activity. Additionally this protein has been described as cytotoxic for the host cell. It is encoded in the left half of the human parvovirus B19 genome. Transcription starts 5′ of the start codon at nucleotide 319 and is driven by the only viral promotor designated P6. The invention relates to the antigenic properties of this protein and to the detection of the genome or transcripts thereof by hybridization with a DNA or RNA strand as a probe in standard hybridization techniques e.g. Southern or Northern blotting or in situ hybridization. The finding of abundant transcripts coding for NS-1 in the infected tissues can be taken as an indicator of deregulation of the genome and an atypical course of infection.

Additionally the invention relates to the cloning of the sequence coding for a protein having the biological activities of NS-1 into another DNA sequence for example the bacterial plasmid pQE40 (Fig. 4, 5) or the baculovirus transfer vector pVL1392 (Fig. 6) and the production of the protein. Insertion of the gene downstream of a promotor allows the expression from the recombinant DNA molecule. Since the NS-1 protein is produced only in trace amounts in the cell infected by the virus efficient production of the protein can only be achieved by recombinant DNA technology. Examples of promotors suited for this purpose are the bacterial lac-promotor of the ß-galactosidase operon for the expression in bacteria, the polyhedrin promotor of baculoviruses for the expression in insect cells or the cytomegalovirus promotor for the production in many types of mammalian cell lines. By means of these constructs the protein can be produced in different hosts e.g. the bacterium E. coli, the yeast Pichia pastoris, Sf-9 insect cells, mammalian or human cell lines like CHO or HeLa cells. For the production of the NS-1 protein these host cells have to be cultered in the appropriate culture media at the specific culture conditions required for the specific host cell. This is for example the cultiviation of E. coli in LB-medium containing antibiotics for the stable maintenance of the expression plasmid. For the E. coli process, the expression is induced by addition of an inducer, usually IPTG for the lac-operon. Afterwards the cells can be harvested by centrifugation, lysed and the protein can be recovered by affinity chromatography or other standard protein purification techniques. Short amino acid sequences of the protein can be obtained by chemical syntheses of peptides. These synthetic peptides may comprise important immunological target sequences. The purified protein or the synthetic peptides can be used for the production of antibodies in rabbits and mice by immunization. Especially the generation of monoclonal antibodies by the hybridoma technique which may be further modified to contain the NS-1 specific V-region fused to human C-domains is of advantage in the current invention. Specific antibodies can be obtained alternatively by the phage display technique.

This invention relates further to the diagnosis, treatment and prevention of the infection with human parvovirus B19. The antibodies or antibody related molecules described above can be used in pharmaceutical compositions for the treatment of diseases caused by the human parvovirus B19. Besides the typically harmless fifth disease, the human parvovirus B19 causes a series of more severe symptoms. An

infection associated arthritis, aplastic crisis in patients with underlying hematopoetic or immunological disorders or immunosuppressed patients and fetal hydrops are the most frequent problems encoutered during infection with human parvovirus B19. Additionally parvovirus B19 has been associated with some cases of meningitis or enterocolitis. Since some of these diseases are associated with the persistence of the virus and the fact that the non-structural protein NS-1 is the most likely protein expressed from the viral genome in such cases, the pharmaceutical composition described above can be used for a therapeutical approach in these patients e.g. by immunoglobulin mediated cure of viral infection. In this context, the mouse-human chimeric antibodies mentioned before are of special interest. Additionally a pharmaceutical composition according to the current invention can be used for vaccination studies to prevent infection with parvovirus B19. This can be done by using this composition for passive immunization of people at risk of exposure. Another part of the invention is the use of the DNA sequences, recombinant proteins or synthetic peptides and the antibodies or antibody related molecules generated by the use of the proteins or peptides and a kit for the diagnosis of infection with human parvovirus B19. The DNA sequences or other probes able to hybridize to the viral genome or transcripts therefrom can be used in a kit for in situ hybridization to bone marrow smears or other tissue samples of the person with suspected infection. Another possibility is the use of such probes in dot-blot tests. Such a kit contains all buffers and probes necessary for successful detection of the viral genome or transcripts thereof as markers for the presence of the virus. The recombinant proteins or synthetic peptides can be used for the detection of specific antibodies in the sera or other body fluids of such persons. This can be done in standard immunological tests e.g. by ELISA test, by RIA, IFT, or an antibody capture assay. The antibodies or antibody like molecules are of great value for the detection of the NS-1 protein in infected tissues by immunoflurescence or demonstration of antigen in body fluids by capture assays e.g. MACRIA.

The use of the sequence coding for the human parvovirus B19 NS-1 protein, the production of the protein itself according to this invention and the use of antibodies or antibody like molecules generated according to this invention greatly facilitate the diagnosis and treatment of an infection with human parvovirus B19.

Description of the drawings

Fig.1: DNA sequence of human parvovirus B19. The left side of the human parvovirus B19 genome sequence from nt 200 to 2700 is shown (Shade et al. 1986). The translation of the NS-1 protein (nt 436-2451) gene is shown below the nucleotide sequence. The NS-1 open reading frame or subfragments corresponding to the amino acid positions 1-303, 150-303, 150-402, 304-492, 403-671, 493-671 were amplified by PCR and inserted into the vector pQE40.

Fig.2: Nucleotide sequences of the primers used for the amplification of different parts of the human parvovirus B19 NS-1 protein. The designations of the amplified sequences are the same as for the protein parts. Underlined nucleotides represent the endonuclease recognition sites used for the insertion of the resulting PCR fragments in the bacterial expression vector pQE40 or the baculovirus transfer vector pVL1392. Nucleotides shown in italics are homologous to the published sequence of the human parvovirus B19 genome (Shade et al., 1986).

Fig.3: Amplification and cloning strategy.
A schematic drawing of the human parvovirus B19 genome is shown on the top. The left part of the human parvovirus B19 genome coding for the NS-1 protein was amplified with different sets of primers. The specific primers used are indicated for each insert (full arrows) Names of the inserts are as listed at the left and the number of the first and last amino acid contained is given. The resulting products were cloned in the plasmid pQE40 by the appropriate restriction endonucleases. The recognition sequences for the enzymes were introduced by the PCR primers and are indicated at the left and right ends of the open reading frames (open boxes: open reading frames; K: *Kpn* I, S: *Sal* I, X: *Xho* I, B: *Bam* HI). The amino terminally fused six histidines are provided by the vector and their positions relative to the inserts are also shown (HIS: histidine linker). VP1/2: open reading frame

coding for the structural proteins, NLS: nuclear localization signal, ATP: ATPase like region.

Fig.4: Expression of recombinant NS-1 protein in procaryotic and eucaryotic cells. SDS-PAGE analysis of the bacterial lysates before (lane 2, -IPTG) and after induction of expression (lane 3, +IPTG). E. coli strain SG13009[pREP4] containing plasmid pQE40NS-1 was grown in LB-medium in the presence of 50µg/ml kanamycin and 100µg/ml ampicillin to an OD_{600} of 0,4 and expression of the recombinant protein was induced by addition of 1mM IPTG for 3 hours. After separation on a SDS-PAGE gel the gel was stained with Coomassie-blue. Clearly a band corresponding to the 71kD NS-1 protein is visible after induction (lane 3: +IPTG). A molecular weight standard is shown at the left side (lane 1: marker), molecular weights are as indicated.

Fig.5: Expression of the NS-1 fragments in E. coli. E. coli strain SG13009[pREP4] was transformed with plasmids pQE40NS/A; pQE40NS/B; pQE40NS/C; pQE40NS/D or pQE40NS/E and pQE40NS/F. Transformed bacteria were grown in LB-medium containing 50µg/ml kanamycin and 100µg/ml ampicillin until an OD_{600} of 0,6 was reached and induced for 5 hours with 1mM IPTG. Bacteria were harvested, lysed in boiling mix (50mM Tris-Cl pH 7,0; 100mM dithiothreitol; 2% SDS; 0,1% bromphenolblue; 10% glycerol) and analyzed by SDS-PAGE and subsequent Coomassie-blue staining. Lane 1: molecular weight marker, lane 2: pQE40NS/A, lane 3: pQE40NS/B, lane 4: pQE40NS/C, lane 5: pQE40NS/D, lane 6: pQE40NS/E, lane 7 pQE40NS/F, lane 8: SG13009 before induction.

Fig.6: Expression of the NS-1 protein in insect cells infected with NS-1 recombinant baculovirus. Sf-9 cells were infected with 0,1 m.o.i. of NS-1 recombinant baculovirus and incubated for four days under standard conditions. The cells were harvested at day four and lysed in boiling mix (50mM Tris-Cl pH 7,0; 100mM dithiothreitol; 2% SDS; 0,1% bromphenolblue; 10% glycerol). The lysates were seperated on a SDS-

PAGE gel and transferred to a nitrocellulose membrane. Detection was done with a NS-1 specific rabbit serum by a standard Western blot procedure.

Fig.7: Demonstration of specific antibodies to parvovirus B19 NS-1 protein in patient sera by immunoblotting.
Recombinantly produced human parvovirus B19 proteins (20-50ng/lane) were separated on a SDS-PAGE gel and transferred to a nitrocellulose membrane. Patient sera were added in a 1:200 dilution. The second antibody was directed against human IgG and coupled to Peroxidase. Serum 382 is from a person not infected previously by B19 parvovirus. This serum does not contain antibodies to the structural proteins VP1 and VP2 (lanes VP1 and VP2 respectively) or the NS-1 protein (lane NS). Serum 377 is from a person with past B19 infection without complications. This serum contains antibodies to the structural proteins but not to NS-1. Serum Ra is from a patient with severe B19 infection associated arthritis. In this probe antibodies to the structural as well as to the NS-1 protein are present.

Fig.8: Detection of NS-1 specific antibodies in human sera by an ELISA:
Microtiter plates were coated with 50ng/well of the recombinant NS-1 protein. The sera were assayed in a 1:100 dilution. A rabbit anti-human IgG serum was used for the detection of specific IgG in the patients sera. Serum 2,10 and Ra, all from patients with a severe B19 associated arthritis are the only probes containing specific antibodies. Sera from previously not infected people or patients with acute or past B19 infection without reported complications did not show any reactivity.

Fig.9: Detection of specific antibodies in patients with persistent infection. Sera collected from two patients with persistent parvovirus B19 infection were investigated. These patients contain specific IgG and IgM antibodies as demonstrated by the ELISA test. Dates of the probe collection are given.

Fig.10: Efficient generation of NS-1 specific antisera in rabbits.

Rabbits were immunized with 50µg of the recombinant NS-1 protein in 250µl Titermax adjuvans and boostered twice with the same composition. Blood was obtained four weeks after the last boost injection and the prepared serum was tested in 1:200 dilution against recombinantly expressed fragments of NS-1 on a Western blot. Detection was done with an alkaline phophatase coupled anti-rabbit antibody. Specific protein bands of the molecular weight corresponding to the NS-1 fragments are recognized by the antibodies in that serum.

Fig. 11: Development of NS-1 protein specific antibody titers.

All three patients developed a strong IgG response specific for NS-1. (A), (B) In patients A and B a good IgM response could be detected whereas in patient (C) the IgM titers remained below 1:128. Titers were determined by end-point dilution and are given as the mean of three independent experiments and reciprocal values of the last reactive serum dilution (solid lines: IgM values, dotted line: IgG values). The time points (month/year) at which serum samples were collected are indicated on the x-axis. Capsid protein specific antibodies were determined as described in the text. Viral DNA was detected by dot-blot or a nested PCR.

<u>The Examples illustrate the invention.</u>

Example 1: Expression of recombinant NS-1 protein in E. coli

To express the recombinant protein in bacteria the E. coli strain SG13009[pREP4] was used. The part of the human parvovirus B19 genome corresponding to the NS-1 open reading frame (Fig. 1) was amplified by a polymerase chain reaction with primers pB19NS-NL (corresponding to nucleotide positions 435-453 in the sequence published by Shade et al. (Shade et al. 1986)) and primer pB19NS-NR (Fig. 2, 3) (corresponding to nucleotide 2430-2451). A *Kpn* I restriction site was introduced via the primer at the 5'-end and a *Xho* I site was generated at the 3'-end the same way (Fig. 1). After 35

cycles amplification at 94°C for 30 sec. / 42°C for 30 sec. / 72°C for 1 min. the resulting 2,0 kB fragment was cut with *Kpn* I and *Xho* I and inserted into the expression vector pQE40 (Diagen GmbH, Germany) cut with *Kpn* I and *Sal* I, using standard molecular cloning procedures as described elsewhere (e.g. Maniatis et al., 1989). This results in the in frame insertion of the NS-1 gene with an amino terminal poly-histidine linker to give plasmid pQE40 NS-1. The plasmids of the pQE series offer the advantage of efficient repression of expression in the absence of an inducer due to the presence of multiple copies of the lac-repressor target sequence upstream of the gene of interest. Additionally the repressor protein is provided in trans by overexpression from a second plasmid [pREP4] in the same host cell, when strain SG13009 is used. The subgenic fragments NS-A, NS-B, NS-C, NS-D, NS-E and NS-F were generated following the same principles (Fig. 5). NS-A had to be expressed as a fusion protein to the mouse DHFR gene for efficient production in this system. The primers used for the amplification of the respective parts of the human parvovirus B19 genome are shown in Fig. 2. After insertion into plasmid pQE40 using the generated restricition sites, plasmids pQE40NS-A, pQE40NS-B, pQE40NS-C, pQE40NS-D, pQE40NS-E and pQE40NS-F were obtained (Fig. 3). Again, in each plasmid the protein is fused to the amino terminal six histidines. The plasmids were transformed into E.coli strain SG13009 and the bacteria were grown in the presence of 100µg/ml ampicillin and 50µg/ml kanamycin until an OD_{600} of 0,4 (for pQE40NS-1) or 0,6 (for other plasmids) were reached. At this OD_{600} the expression of the recombinant proteins was induced by addition of 1mM IPTG. The growth temperature for efficient expression of the complete protein had to be lowered to 25°C. 5 hours post induction the cells were harvested by centrifugation at 5000 Upm for 15 min. (Kontron Centrikon T-124, rotor A 6.14)

Example 2: Purification of the recombinant protein.

For the purification of the bacterially expressed proteins, the protocol for insoluble proteins as suggested by the manufacturer was used (The QIAexpressionist; 2nd edition, 1992; Diagen GmbH).

Briefly, the supernatant was discarded after centrifugation and the bacterial pellet was lysed in a buffer consisting of 8M urea/ 100mM Na-phosphate/ 10mM Tris-HCl pH 8,0 (buffer A) for 30 min. at room temperature and subsequent sonification with three pulses of 60 sec. at 200 W on ice. The lysate was centrifuged at 10.000 Upm for 15 min. in a high speed centrifuge (Kontron Centrikon T-124; rotor A 8.24). The supernatant of a 1 litre culture was loaded on a 1,5ml Ni-Agarose column (15mm diameter) equilibrated with buffer A and adsorbed at a flow rate of approximately 0,1 ml/min. The column was washed with 10 volumes buffer A and afterwards with 20 volumes buffer B (8 M urea / 0,1 M Na-phosphate / 10 mM Tris-HCl pH 6,3) and buffer C (8 M urea / 0,1 M Na-phosphate / 10mM Tris-HCl pH 5,9). Subsequently the proteins were eluted with 0,2 M Imidazol in buffer B. Alternatively the pH of buffer C was lowered to pH 4,5 which was sufficient for elution of the recombinant proteins. All steps were carried out at room temperature in the presence of proteinase inhibitors (1mM PMSF; 1mM E64 (Boehringer Mannheim GmbH)). The peak fractions were determined by SDS-PAGE analysis (Laemmli, 1970) and subsequent Coomassie-blue staining of the gel. The specificity of the eluted protein was tested by electrophoretic transfer of the proteins on a nitrocellulose membrane (Western blotting) and detection with NS-1 specific rabbit antisera according to standard procedures (Maniatis et al., 1989).The peak fractions were pooled and loaded on a preparative SDS-PAGE column for further purification.

This was done using a Prep-Cell® device (BioRad GmbH, Germany). The resolution gel concentrations were 10% acrylamide/bisaycrylamide (29:1) for the whole protein and 12,5% for the smaller proteins. The column consisted of a 1cm stacking gel

and a 6,5 cm resolution gel and was run at 40mA. The collection of fractions was started when the running front of the probe reached the lower end of the column. 2,5 ml fractions were collected and the protein peak was localized by measuring the UV light adsorption of the solution. The peak fractions were analyzed exactly as described above for the Ni-Agarose column.

Example 3: Detection of antibodies against the recombinant proteins in humans

The purified protein can be used to detect antibodies against the NS-1 protein in humans infected with the human parvovirus B19. For this purpose, we employed two different techniques.

I. Detection of antibodies against the NS-1 protein of human parvovirus B19 by Immunoblots (Fig. 7).

The purified proteins (appr. 50ng) were run on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane according to standard protocols (Towbin et al., 1979). Subsequently the membrane was blocked with 5% low-fat dry milk solubilized in Tris-buffered saline pH 7,5 for 1 hour at room temperature and incubated with the patients sera diluted 1:200 in Tris-buffered saline pH 7,5 overnight at room temperature. The membranes were washed three times with Tris-buffered saline pH 7,5 / 0,5% TWEEN 20 for 15 min. at room temperature before the second antibody was added. The second antibody was a Peroxidase coupled anti-human µ-chain antiserum raised in rabbits (P215; DAKO GmbH, Germany) or an alkaline phosphatase coupled rabbit antiserum to human IgG γ-chains (BioRad GmbH, Germany). After incubation at room temperature for 2-3 hours the blots were washed again with Tris-buffered saline pH 7,5/ 0,5% TWEEN 20 and, for the alkaline phosphatase staining, equilibrated in 0,2M Na-carbonate buffer pH 9,2 / 5mM $MgCl_2$ for 10 min., room temperature. Colour development was done according to standard procedure using diaminobenzidine for the

peroxidase and BCIP/ NBT as substrate for the alkaline phosphatase (Maniatis et al., 1989). When sera of people with typical *erythema infectiosum*, passed human parvovirus B19 infection, severe human parvovirus B19 associated arthritis or persistent infection were tested, surprisingly only the arthritis patients (Fig. 7, 8) and the persistently (Fig. 9) infected individuals displayed a specific reaction with the NS-1 protein. This is apparent by the staining of a specific band with a molecular weight of the recombinant protein. These findings are confirmed if the reactivity against different fragments of the protein are investigated.

II. Detection of antibodies against the NS-1 protein of human parvovirus B19 by ELISA.

To demonstrate specific antibodies for the human parvovirus B19 NS-1 protein by ELISA, reaction plates (Greiner flat bottom F plates; medium binding capacity) were coated with 50ng of the purified recombinant protein in a total volume of 50µl per well in 0,2M Na-carbonate buffer pH9,2 / 0,15M NaCl overnight at 37°C in a wet chamber. The plates were washed 5 times with 200µl/well PBS / 0,9% NaCl / 0,5% TWEEN 20 before 50µl/well of the sera diluted in PBS /0,3% FCS / 0,2% TWEEN 20 were added. After 3 hours incubation at 37°C in a wet chamber the plates were again washed 5 times with 200µl/well PBS / 0,9% NaCl / 0,5% TWEEN 20 and specific IgG or IgM class antibodies were detected by addition of 50µl/well of the second antibodies diluted 1:1000 in the same buffer as the patient sera (Fig. 8, 9). Second antibodies used were a peroxidase coupled rabbit anti-human µ-chain serum (P215, DAKO GmbH, Germany) or a peroxidase conjugated rabbit anti-human γ-chain serum (P214, DAKO GmbH, Germany). Colour development was done with o-phenylendiamine as substrate (100µl/well) for 10 min. at room temperature, stopped with 100µl 2N H_2SO_4 and the OD_{492} was measured in an ELISA reader (Fig. 8, 9).

Example 4: Production of NS-1 specific antisera

Four rabbits (New Zealand White; 4kg weight; males; obtained from Charles River Wega, Germany) were inoculated with 50µg of recombinant NS-1 protein emulgated in 250µl of Titermax® Adjuvans (Serva GmbH, Germany) at the lower chest s.c.. Four weeks and 3 months after the intial immunization, two boost injections were given essentially composed like the first one. Blood was drawn from the ear at different time points p.i. and the sera tested for specific antibodies against NS-1 and different purified subfragments (Fig. 10). Titers of reactive sera were determined by ELISA. ELISA and Western blots were carried out exactly like described above for human sera. All rabbits developed a strong immune response after the second boost injection.

Example 5: Correlation of NS-1 specific antibodies in clinical cases of B19 infection

Three patients with prolonged or persistent parvovirus B19 infection were identified. Patient A, a 77 year old female, suffered from recurrent granulocytic aplasia and has been described previously (Pont et al., 1992).

Patient B, a 59 year old female, was treated with cytostatic drugs (Leukeran®, Wellcome Found., London, England; Aprednislon®, Merck, Vienna, Austria; Sterecyt®, Kabi Pharmacia, Uppsala, Sweden) because of chronic B-cell lymphatic leucemia (b-CLL) with autoimmune hemolytic anemia and position Coombs test. Erythrocyte counts remained stable (Hb: 11.0g/dL). After interruption of cytostatic treatment due to the onset of severe Cytomegalovirus (CMV) infection, massive hemolysis with low reticulocyte counts was observed and parvovirus B19 IgM antibodies directed to the capsid proteins, as well as viral DNA, became detectable for the first time. After two months, erythrocyte counts had markedly improved. About

half a year later a renewed significant transient drop of red cell counts was observed.

Patient C, a 23 year old woman, presented with fever, pancytopenia and complete bone marrow aplasia. A moderate elevation of white cell counts was observed, but erythrocyte numbers remained low (Hb: 9.3g/dL). At this time IgM and IgG to parvovirus B19 capsid proteins as well as viral DNA became detectable. Red cell counts improved after substitution or erythrocytes and ATG treatment (Presimmun®, Behring AG, Germany). In parallel Hepatitis -C-Virus (HCV) infection was diagnosed by PCR analysis about eight weeks after parvovirus infection.

Sera were tested for the presence of antibodies to parvovirus B19 capsid proteins VP1 and VP2 by different capture ELISA tests using isolated B19 virions and a monoclonal antibody to parvovirus B19 (VRL/B19/11) (Schwarz et al., 1988). Confirmative testing for patient A by RIA was performed by B.J. Choen, Virus Reference Laboratory, London.

Antibodies to the non-structural protein NS-1 were tested retrospectively with an ELISA based on recombinant protein as described in Example 3 and in Poblotzki et al., 1995.

Viral DNA was detected by dot-blot analysis as described previously (Pont et al., 1988). Briefly 5µl of serum were dotted on a nylon membrane and detected according to the manufacturers protocol using digoxigenin labelled 700bp DNA fragments from the viral genome as hybridization probe (Boehringer Mannheim GmbH, Penzberg, Germany). This test allows detection of virus concentrations with more than 10^7 particles per mL serum. A nested PCR assay was set up using pairs of primers from the region of the B19 genome coding for the capsid proteins (outer primers nt 2901-

2918 and 3511-3529; inner primers nt 2956-2972 and 3431-3448). 40 cycles of 30 seconds at 94°C, 1 minute at 45°C and extension at 72°C for 1 minute for the first and second amplification were done (Perkin Elmer 9600 Cycler). Reaction products were separated on agarose gels and DNA was visualized by staining with ethidium bromide. Using this assay we were able to detect 100 viral particles as determined by serial ten-fold dilutions of cloned B19 DNA in human serum as well as with a viraemic serum obtained according to conventional procedures.

Patient A has NS-1 specific antibodies present in serum samples drawn after a first admission to the hospital due to an anemia without significant impairment of the white cell counts. She also had IgM and IgG specific for B19 capsid protein at this time (Fig. 11, patient A). At renewed admission ten months later severe granulocytopenia without significant impairment of red cell counts was found (Pont et al., 1988). Concomitantly the patient developed a strong rise in the IgG response to NS-1 protein. After intravenous treatment of the infection with high-titered immunoglobulins to the capsid proteins, IgM titers to NS-1 rapidly declined while IgG remained detectable. In contrast, the antibodies against the capsid proteins remained detectable for about four weeks only (Fig. 11A; patient A VII/89). After four months virus-specific DNA was still present in the serum, but neither IgM nor IgG directed to the capsid protein were found. A second period of severe agranulocytosis was accompanied by a moderate rise of NS-1 specific IgG but not IgM and was treated again with immunoglobulin (Fig. 11A; patient A VI/90). This lead to the development of a stable capsid protein specific IgG response and the disappearance of viral DNA from the serum.

Patient B, whose immunosuppressive treatment was interrupted because of a severe CMV infection, developed a strong hemolytic syndrome with accompanying low reticulocyte counts. In parallel to

antibodies directed against the capsid proteins, high titers of IgM and IgG directed to the NS-1 protein were detectable in the first serum sample (Fig. 11B; patient B). One month later parvovirus B19 DNA was detectable in the serum by dot-blot hybridization. In parallel to the improvement of the clinical condition, titers of NS-1 specific IgM titers and a more pronounced elevation of specific IgG was detected. At this time a renewed drop in the red cell counts was observed; three weeks later B19 DNA was found again in serum by PCR but had disappeared after two months. Impairment of the granulocyte numbers was difficult to assess due to the B-CLL.

Patient C developed antibodies specific to the parvovirus B19 capsid proteins during a period of complete bone marrow aplasia and febrile illness. No significant impairment of the white cells was found, viral DNA was detectable by hybridization for an unusually long time of about four weeks and remained detectable by nested PCR. The IgM titer specific for the NS-1 protein was only 1:64 but IgG was clearly detectable. After resolution of the aplasia, IgM specific for capsid proteins disappeared. Several months later, a rise in NS-1 specific IgG and also a moderate elevation of the IgM concentrations were observed (Fig. 11C; patient C XI/90) without obvious clinical manifestations.

NS-1 specific antibodies were not observed in a panel of control sera from patients with acute *erythema infectiosum*. Even in sera taken directly after the onset of disease that contained IgM and IgG specific for the capsid proteins, antibodies specific for the NS-1 protein were not detected. Additionally, we could not find NS-1 specific antibodies in sera from patients with serological evidence for past infections or without previous exposure to parvovirus B19.

All three patients described in this example suffered from severe, obviously persistent parvovirus B19 infection. Only one of them

(patient B) had been known to be immunocompromised before the onset of disease which makes the basis of persistence difficult to explain. So far, no abnormalities of the immune system could be found besides the initial failure in patients A and B to generate a stable IgG response to the capsid proteins. In all three cases parvovirus B19 replication was indicated by the prolonged presence of high concentrations of viral DNA in the sera, that were detectable by dot-blot hybridization. In contrast to sera from patients with acute *erythema infectiosum* these patients developed a humoral immune response to the NS-1 protein. Patients A and B have high IgM titers at the beginning of infection, whereas patient C displayed only low specific IgM values (Fig. 11). For this case there was evidence for the presence of viral DNA in a serum sample drawn about six months earlier, which unfortunately was not available for this study. This could mean, that the IgM response has declined already in the first sample investigated here.

The generation of NS-1 specific antibodies may reflect an elevated production of this protein in the cases described. As a consequence of the prolonged viral presence in the circulation, non-erythroid cells bearing the receptor for parvovirus B19 but normally less susceptible for infection might become infected (Brown et al., 1993). In the non-permissive cells the regulation of viral gene expression is strongly shifted towards preferential production of NS-1 protein that is cytotoxic (Lin et al., 1992; Ozawa et al., 1988). The abortive infection of these cells would have two consequences: an elevated synthesis of NS-1 protein and its release after cell death. This would result in efficient induction of an NS-1 specific humoral immune response. However, if the cells destroyed by abortive infection are leucocytes or synovial cells, it would explain the clinically observed manifestations in some cases of B19 infection. The exclusive observation of NS-1 specific immunoglobulins in persistently infected persons with impairment of white cell counts or pancytopenia,

together with the data presented in Example 3, namely that NS-1 specific antibodies in patients with B19-associated arthritis is in agreement with the hypothesis that the non-structural protein plays an important role in cytotoxicity of parvovirus B19 in non-permissive cells.

The onset of granulocytopenia and thrombocytopenia in patient A was accompanied by a rise of NS-1 specific IgG titers, whereas in patient B worsening of symptoms correlated with an elevation of NS-1 specific IgM and IgG titers and recurrence of viral DNA. This correlation could reflect the renewed synthesis of NS-1 protein in infected cells after reactivation of viral gene expression resulting in a boost effect on antibody production. It supports the hypothesis of a putative involvement of this protein in pathogenesis of granulocytopenia and thrombocytopenia in the patients described.

In contrast to NS-1 specific IgG, antibodies to the capsid proteins VP1 and VP2 were not detectable in patients at all time points of investigation. Therefore we suggest that analysis of NS-1 specific antibodies could help to confirm the role of parvovirus B19 in cytopenias or bone marrow aplasia, particularly since viral DNA is often difficult to detect in sera from those persons. Furthermore, the data presented in the Examples emphasis the role of NS-1 specific antibodies as a useful means for the detection of a prolonged or persistent B19 infection.

Example 6: Haemophiliacs with antibodies against parvovirus B19

A collective of 20 haemophiliacs with IgM against the structural proteins of parvovirus B19 that was serologically detectable over a longer period of time was tested against antibodies against the NS-1 protein. In none of these patients anti-NS-1 protein antibodies could be detected. Further, viral DNA could not be detected. The conclusion from these data in these cases is that the B19 parvovirus

infection is not a persistent infection. A possible explanation for the occurrence of the prolonged IgM immune response is the repeated transfusion of blood or the administration of blood preparations. Accordingly, the test for NS-1 specific antibodies can, in such cases facilitate an exclusion diagnosis.

Example 7: Correlation between persistent B19 infection and Wegener's granulomatosis

Recently, Wegener's granulomatosis was indicated to be correlated with persistent parvovirus B19 infection.

In accordance with the present invention eight cases of Wegener's granulomatosis were investigated. In no case specific antibodies against NS-1 or viral DNA could be detected. Accordingly, also in this case exclusion diagnosis can be facilitated.

Example 8: Statistics of correlation of antibodies specific for NS-1 and B19 infection

On the whole, 130 patient with a previous parvovirus B19 infection, 31 patients with an acute parvovirus B19 infection and 39 seronegative patients were tested for antibodies against NS-1 protein. In the case of the patients with acute infections, the criterion for the acuteness of the infection was detectable IgM antibodies against the structural proteins in the serum. In none of the 200 aforementioned persons antibodies against NS-1 could be detected using the methods described herein above. Again, this shows the specificity of the detection system for NS-1 specific antibodies described above for cases of prolonged or persisting parvovirus B19 infection.

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Claims

1. A DNA sequence coding for a protein having the biological activities of the non-structural protein NS-1 of the human parvovirus B19, said DNA sequence being selected from the group consisting of:

   a) a DNA sequence encoding the protein given in figure 1;
   b) the DNA sequence given in figure 1 from nucleotide 319 to 2645;
   c) a DNA sequence able to hybridize to a DNA sequence of a) and b).

2. A probe derived from a DNA sequence according to claim 1 which is capable of selectively hybridizing to the genome of human parvovirus B19, or transcripts therefrom.

3. A recombinant DNA molecule containing a DNA sequence according to claim 1.

4. The recombinant DNA molecule of claim 2, wherein the DNA sequence is under control of a functional promotor.

5. A procaryotic or eucaryotic host cell containing a recombinant DNA molecule according to claim 3 or 4.

6. A process for the production of a protein having the biological activities of non-structural protein NS-1 of the human parvovirus B19, comprising the steps of cultivating the host cell according to claim 5 and recovering the protein from culture.

7. A protein or peptide having the biological activities of the non-structural protein NS-1 of the human parvovirus B19 which is encoded by the DNA sequence of claim 1 or which is obtained by the process of claim 6.

8. The peptide of claim 7 bearing an epitope of the non-structural protein NS-1 of the human parvovirus B19.

9. An antibody or antibody derivative which specifically recognizes a protein or peptide according to claim 7 or 8.

10. The antibody according to claim 9 which is a monoclonal antibody.

11. A pharmaceutical composition containing a protein or peptide according to claim 7 or 8 or an antibody or antibody derivative according to claim 9 or 10, optionally in combination with a pharmaceutically acceptable carrier and/or diluent.

12. The pharmaceutical composition according to claim 11 for the treatment of diseases caused by infection with the human parvovirus B19.

13. The pharmaceutical composition according to claim 11 for the prophylaxis of infections with the human parvovirus B19.

14. A kit for the detection of diseases caused by infections with the human parvovirus B19 that contains:

    a) at least one of the DNA sequences or probes according to claim 1 or 2; and/or
    b) at least one of the proteins or peptides according to claim 7 or 8; and/or
    c) at least one of the antibodies or antibody dervatives of claims 9 or 10.

15. The pharmaceutical composition according to any one of claims 11 to 13 or the kit according to claim 14 wherein the infection is prolonged or persistent infection with human parvovirus B19.

16. Use of the kit according to claim 14 for the exclusion diagnosis of prolonged or persistent parvovirus B19 infection.

SUMMARY:

DNA sequences of the human parvovirus B19 genome coding for the non-structural protein, recombinant DNA molecules containing such sequences, antigens coded within these sDNA sequences of the parvovirus B19 genome coding for the non-structural protein sequences, production of those antigens, generation of antibodies or antibody related molecules directed to such antigens, use of these sequences, antigens, antibodies or antibody related molecules for diagnostic, therapeutic or vaccination purposes.

Fig.1: Sequence of the human parvovirus B19.

```
           GAATTCCGCCAAATCAGATGCCGCCGGTCGCCGCCGGTAGGCGGGACTTCCGGTACAAGA
         1 ---------+---------+---------+---------+---------+---------+ 60
           CTTAAGGCGGTTTAGTCTACGGCGGCCAGCGGCGGCCATCCGCCCTGAAGGCCATGTTCT

a          E  F  R  Q  I  R  C  R  R  S  P  P  V  G  G  T  S  G  T  R  -

           TGGCGGACAATTACGTCATTTCCTGTGACGTCATTTCCTGTGACGTCACAGGAAATGACG
        61 ---------+---------+---------+---------+---------+---------+ 120
           ACCGCCTGTTAATGCAGTAAAGGACACTGCAGTAAAGGACACTGCAGTGTCCTTTACTGC

a          W  R  T  I  T  S  F  P  V  T  S  F  P  V  T  S  Q  E  M  T  -

           TAATTGTCCGCCATCTTGTACCGGAAGTCCCGCCTACCGGCGGCGACCGGCGGCATCTGA
       121 ---------+---------+---------+---------+---------+---------+ 180
           ATTAACAGGCGGTAGAACATGGCCTTCAGGGCGGATGGCCGCCGCTGGCCGCCGTAGACT

a          *                                                          -

           TTTGGTGTCTTCTTTTAAATTTTAGCGGGCTTTTTTCCCGCCTTATGCAAATGGGCAGCC
       181 ---------+---------+---------+---------+---------+---------+ 240
           AAACCACAGAAGAAAATTTAAAATCGCCCGAAAAAAGGGCGGAATACGTTTACCCGTCGG

a                                                                     -

           ATTTTAAGTGTTTTACTATAATTTTATTGGTTAGTTTTGTAACGGTTAAAATGGGCGGAG
       241 ---------+---------+---------+---------+---------+---------+ 300
           TAAAATTCACAAAATGATATTAAAATAACCAATCAAAACATTGCCAATTTTACCCGCCTC

a                                                                     -

           CGTAGGCGGGGACTACAGTATATATAGCACGGTACTGCCGCAGCTCTTTCTTTCTGGGCT
       301 ---------+---------+---------+---------+---------+---------+ 360
           GCATCCGCCCCTGATGTCATATATATCGTGCCATGACGGCGTCGAGAAAGAAAGACCCGA

a                                                                     -

           GCTTTTTCCTGGACTTTCTTGCTGTTTTTTGTGAGCTAACTAACAGGTATTTATACTACT
       361 ---------+---------+---------+---------+---------+---------+ 420
           CGAAAAAGGACCTGAAAGAACGACAAAAAACACTCGATTGATTGTCCATAAATATGATGA

a                                                                     -

           TGTTAACATCCTAACATGGAGCTATTTAGAGGGGTGCTTCAAGTTTCTTCTAATGTTCTA
       421 ---------+---------+---------+---------+---------+---------+ 480
           ACAATTGTAGGATTGTACCTCGATAAATCTCCCCACGAAGTTCAAAGAAGATTACAAGAT

a                         M  E  L  F  R  G  V  L  Q  V  S  S  N  V  L  -

           GACTGTGCTAACGATAACTGGTGGTGCTCTTTACTGGATTTAGACACTTCTGACTGGGAA
       481 ---------+---------+---------+---------+---------+---------+ 540
           CTGACACGATTGCTATTGACCACCACGAGAAATGACCTAAATCTGTGAAGACTGACCCTT

a          D  C  A  N  D  N  W  W  C  S  L  L  D  L  D  T  S  D  W  E  -

           CCACTAACTCATACTAACAGACTAATGGCAATATACTTAAGCAGTGTGGCTTCTAAGCTT
       541 ---------+---------+---------+---------+---------+---------+ 600
           GGTGATTGAGTATGATTGTCTGATTACCGTTATATGAATTCGTCACACCGAAGATTCGAA

a          P  L  T  H  T  N  R  L  M  A  I  Y  L  S  S  V  A  S  K  L  -

           GACTTTACCGGGGGGCCACTAGCAGGGTGCTTGTACTTTTTTCAAGTAGAATGTAACAAA
       601 ---------+---------+---------+---------+---------+---------+ 660
           CTGAAATGGCCCCCCGGTGATCGTCCCACGAACATGAAAAAAGTTCATCTTACATTGTTT

a          D  F  T  G  G  P  L  A  G  C  L  Y  F  F  Q  V  E  C  N  K  -
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         TTTGAAGAAGGCTATCATATTCATGTGGTTACTGGGGGGCCAGGGTTAAACCCCAGAAAC
    661  ---------+---------+---------+---------+---------+---------+ 720
         AAACTTCTTCCGATAGTATAAGTACACCAATGACCCCCCGGTCCCAATTTGGGGTCTTTG

a        F  E  E  G  Y  H  I  H  V  V  T  G  G  P  G  L  N  P  R  N  -

         CTTACAGTGTGTGTAGAGGGGTTATTTAATAATGTACTTTATCACCTTGTAACTGAAAAT
    721  ---------+---------+---------+---------+---------+---------+ 780
         GAATGTCACACACATCTCCCCAATAAATTATTACATGAAATAGTGGAACATTGACTTTTA

a        L  T  V  C  V  E  G  L  F  N  N  V  L  Y  H  L  V  T  E  N  -

         GTGAAGCTAAAATTTTTGCCAGGAATGACTACAAAAGGCAAATACTTTAGAGATGGAGAG
    781  ---------+---------+---------+---------+---------+---------+ 840
         CACTTCGATTTTAAAAACGGTCCTTACTGATGTTTTCCGTTTATGAAATCTCTACCTCTC

a        V  K  L  K  F  L  P  G  M  T  T  K  G  K  Y  F  R  D  G  E  -

         CAGTTTATAGAAAACTATTTAATGAAAAAAATACCTTTAAATGTTGTATGGTGTGTTACT
    841  ---------+---------+---------+---------+---------+---------+ 900
         GTCAAATATCTTTTGATAAATTACTTTTTTTATGGAAATTTACAACATACCACACAATGA

a        Q  F  I  E  N  Y  L  M  K  K  I  P  L  N  V  V  W  C  V  T  -

         AATATTGATGGATATATAGATACCTGTATTTCTGCTACTTTTAGAAGGGGAGCTTGCCAT
    901  ---------+---------+---------+---------+---------+---------+ 960
         TTATAACTACCTATATATCTATGGACATAAAGACGATGAAAATCTTCCCCTCGAACGGTA

a        N  I  D  G  Y  I  D  T  C  I  S  A  T  F  R  R  G  A  C  H  -

         GCCAAGAAACCCCGCATTACCACAGCCATAAATGATACTAGTAGTGATGCTGGGGAGTCT
    961  ---------+---------+---------+---------+---------+---------+ 1020
         CGGTTCTTTGGGGCGTAATGGTGTCGGTATTTACTATGATCATCACTACGACCCCTCAGA

a        A  K  K  P  R  I  T  T  A  I  N  D  T  S  S  D  A  G  E  S  -

         AGCGGCACAGGGGCAGAGGTTGTGCCATTTAATGGGAAGGGAACTAAGGCTAGCATAAAG
   1021  ---------+---------+---------+---------+---------+---------+ 1080
         TCGCCGTGTCCCCGTCTCCAACACGGTAAATTACCCTTCCCTTGATTCCGATCGTATTTC

a        S  G  T  G  A  E  V  V  P  F  N  G  K  G  T  K  A  S  I  K  -

         TTTCAAACTATGGTAAACTGGTTGTGTGAAAACAGAGTGTTTACAGAGGATAAGTGGAAA
   1081  ---------+---------+---------+---------+---------+---------+ 1140
         AAAGTTTGATACCATTTGACCAACACACTTTTGTCTCACAAATGTCTCCTATTCACCTTT

a        F  Q  T  M  V  N  W  L  C  E  N  R  V  F  T  E  D  K  W  K  -

         CTAGTTGACTTTAACCAGTACACTTTACTAAGCAGTAGTCACAGTGGAAGTTTTCAAATT
   1141  ---------+---------+---------+---------+---------+---------+ 1200
         GATCAACTGAAATTGGTCATGTGAAATGATTCGTCATCAGTGTCACCTTCAAAAGTTTAA

a        L  V  D  F  N  Q  Y  T  L  L  S  S  S  H  S  G  S  F  Q  I  -

         CAAAGTGCACTAAAACTAGCAATTTATAAAGCAACTAATTTAGTGCCTACTAGCACATTT
   1201  ---------+---------+---------+---------+---------+---------+ 1260
         GTTTCACGTGATTTTGATCGTTAAATATTTCGTTGATTAAATCACGGATGATCGTGTAAA

a        Q  S  A  L  K  L  A  I  Y  K  A  T  N  L  V  P  T  S  T  F  -

         TTATTGCATACAGACTTTGAGCAGGTTATGTGTATTAAAGACAATAAAATTGTTAAATTG
   1261  ---------+---------+---------+---------+---------+---------+ 1320
         AATAACGTATGTCTGAAACTCGTCCAATACACATAATTTCTGTTATTTTAACAATTTAAC

a        L  L  H  T  D  F  E  Q  V  M  C  I  K  D  N  K  I  V  K  L  -
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       TTACTTTGTCAAAACTATGACCCCCTATTGGTGGGGCAGCATGTGTTAAAGTGGATTGAT
  1321 ---------+---------+---------+---------+---------+---------+ 1380
       AATGAAACAGTTTTGATACTGGGGGATAACCACCCCGTCGTACACAATTTCACCTAACTA

a      L  L  C  Q  N  Y  D  P  L  L  V  G  Q  H  V  L  K  W  I  D  -

       AAAAAATGTGGTAAGAAAAATACACTGTGGTTTTATGGGCCGCCAAGTACAGGAAAAAACA
  1381 ---------+---------+---------+---------+---------+---------+ 1440
       TTTTTTACACCATTCTTTTTATGTGACACCAAAATACCCGGCGGTTCATGTCCTTTTTTGT

a      K  K  C  G  K  K  N  T  L  W  F  Y  G  P  P  S  T  G  K  T  -

       AACTTGGCAATGGCCATTGCTAAAAGTGTTCCAGTATATGGCATGGTTAACTGGAATAAT
  1441 ---------+---------+---------+---------+---------+---------+ 1500
       TTGAACCGTTACCGGTAACGATTTTCACAAGGTCATATACCGTACCAATTGACCTTATTA

a      N  L  A  M  A  I  A  K  S  V  P  V  Y  G  M  V  N  W  N  N  -

       GAAAACTTTCCATTTAATGATGTAGCAGGAAAAAGCTTGGTGGTCTGGGATGAAGGTATT
  1501 ---------+---------+---------+---------+---------+---------+ 1560
       CTTTTGAAAGGTAAATTACTACATCGTCCTTTTTCGAACCACCAGACCCTACTTCCATAA

a      E  N  F  P  F  N  D  V  A  G  K  S  L  V  V  W  D  E  G  I  -

       ATTAAGTCTACAATTGTAGAAGCTGCAAAAGCCATTTTAGGCGGGCAACCCACCAGGGTA
  1561 ---------+---------+---------+---------+---------+---------+ 1620
       TAATTCAGATGTTAACATCTTCGACGTTTTCGGTAAAATCCGCCCGTTGGGTGGTCCCAT

a      I  K  S  T  I  V  E  A  A  K  A  I  L  G  G  Q  P  T  R  V  -

       GATCAAAAAATGCGTGGAAGTGTAGCTGTGCCTGGAGTACCTGTGGTTATAACCAGCAAT
  1621 ---------+---------+---------+---------+---------+---------+ 1680
       CTAGTTTTTTACGCACCTTCACATCGACACGGACCTCATGGACACCAATATTGGTCGTTA

a      D  Q  K  M  R  G  S  V  A  V  P  G  V  P  V  V  I  T  S  N  -

       GGTGACATTACTTTTGTTGTAAGCGGGAACACTACAACAACTGTACATGCTAAAGCCTTA
  1681 ---------+---------+---------+---------+---------+---------+ 1740
       CCACTGTAATGAAAACAACATTCGCCCTTGTGATGTTGTTGACATGTACGATTTCGGAAT

a      G  D  I  T  F  V  V  S  G  N  T  T  T  T  V  H  A  K  A  L  -

       AAAGAGCGCATGGTAAAGTTAAACTTTACTGTAAGATGCAGCCCTGACATGGGGTTACTA
  1741 ---------+---------+---------+---------+---------+---------+ 1800
       TTTCTCGCGTACCATTTCAATTTGAAATGACATTCTACGTCGGGACTGTACCCCAATGAT

a      K  E  R  M  V  K  L  N  F  T  V  R  C  S  P  D  M  G  L  L  -

       ACAGAGGCTGATGTACAACAGTGGCTTACATGGTGTAATGCACAAAGCTGGGACCACTAT
  1801 ---------+---------+---------+---------+---------+---------+ 1860
       TGTCTCCGACTACATGTTGTCACCGAATGTACCACATTACGTGTTTCGACCCTGGTGATA

a      T  E  A  D  V  Q  Q  W  L  T  W  C  N  A  Q  S  W  D  H  Y  -

       GAAAACTGGGCAATAAACTACACTTTTGATTTCCCTGGAATTAATGCAGATGCCCTCCAC
  1861 ---------+---------+---------+---------+---------+---------+ 1920
       CTTTTGACCCGTTATTTGATGTGAAAACTAAAGGGACCTTAATTACGTCTACGGGAGGTG

a      E  N  W  A  I  N  Y  T  F  D  F  P  G  I  N  A  D  A  L  H  -

       CCAGACCTCCAAACCACCCCAATTGTCACAGACACCAGTATCAGCAGCAGTGGTGGTGAA
  1921 ---------+---------+---------+---------+---------+---------+ 1980
       GGTCTGGAGGTTTGGTGGGGTTAACAGTGTCTGTGGTCATAGTCGTCGTCACCACCACTT

a      P  D  L  Q  T  T  P  I  V  T  D  T  S  I  S  S  S  G  G  E  -
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        AGCTCTGAAGAACTCAGTGAAAGCAGCTTTTTAACCTCATCACCCCAGGCGCCTGGAAC
   1981 ---------+---------+---------+---------+---------+---------+ 2040
        TCGAGACTTCTTGAGTCACTTTCGTCGAAAAATTGGAGTAGTGGGGTCCGCGGACCTTG

a        S  S  E  E  L  S  E  S  S  F  F  N  L  I  T  P  G  A  W  N   -

        ACTGAAACCCCGCGCTCTAGTACGCCCATCCCCGGGACCAGTTCAGGAGAATCATTTGTC
   2041 ---------+---------+---------+---------+---------+---------+ 2100
        TGACTTTGGGGCGCGAGATCATGCGGGTAGGGGCCCTGGTCAAGTCCTCTTAGTAAACAG

a        T  E  T  P  R  S  S  T  P  I  P  G  T  S  S  G  E  S  F  V   -

        GGAAGCCCAGTTTCCTCCGAAGTTGTAGCTGCATCGTGGGAAGAAGCCTTCTACACACCT
   2101 ---------+---------+---------+---------+---------+---------+ 2160
        CCTTCGGGTCAAAGGAGGCTTCAACATCGACGTAGCACCCTTCTTCGGAAGATGTGTGGA

a        G  S  P  V  S  S  E  V  V  A  A  S  W  E  E  A  F  Y  T  P   -

        TTGGCAGACCAGTTTCGTGAACTGTTAGTTGGGGTTGATTATGTGTGGGACGGTGTAAGG
   2161 ---------+---------+---------+---------+---------+---------+ 2220
        AACCGTCTGGTCAAAGCACTTGACAATCAACCCCAACTAATACACACCCTGCCACATTCC

a        L  A  D  Q  F  R  E  L  L  V  G  V  D  Y  V  W  D  G  V  R   -

        GGTTTACCTGTGTGTTGTGTGCAACATATTAACAATAGTGGGGGAGGGTTGGGACTTTGT
   2221 ---------+---------+---------+---------+---------+---------+ 2280
        CCAAATGGACACACAACACACGTTGTATAATTGTTATCACCCCCTCCCAACCCTGAAACA

a        G  L  P  V  C  C  V  Q  H  I  N  N  S  G  G  G  L  G  L  C   -

        CCCCATTGCATTAATGTAGGGGCTTGGTATAATGGATGGAAATTTCGAGAATTTACCCCA
   2281 ---------+---------+---------+---------+---------+---------+ 2340
        GGGGTAACGTAATTACATCCCCGAACCATATTACCTACCTTTAAAGCTCTTAAATGGGGT

a        P  H  C  I  N  V  G  A  W  Y  N  G  W  K  F  R  E  F  T  P   -

        GATTTGGTGCGATGTAGCTGCCATGTGGGAGCTTCTAATCCCTTTTCTGTGCTAACCTGC
   2341 ---------+---------+---------+---------+---------+---------+ 2400
        CTAAACCACGCTACATCGACGGTACACCCTCGAAGATTAGGGAAAAGACACGATTGGACG

a        D  L  V  R  C  S  C  H  V  G  A  S  N  P  F  S  V  L  T  C   -

        AAAAAATGTGCTTACCTGTCTGGATTGCAAAGCTTTGTAGATTATGAGTAAAAAAAGTGG
   2401 ---------+---------+---------+---------+---------+---------+ 2460
        TTTTTTACACGAATGGACAGACCTAACGTTTCGAAACATCTAATACTCATTTTTTTCACC

a        K  K  C  A  Y  L  S  G  L  Q  S  F  V  D  Y  E  *            -

        CAAATGGTGGGAAAGTGATGATAAATTTGCTAAAGCTGTGTATCAGCAATTTGTGGAATT
   2461 ---------+---------+---------+---------+---------+---------+ 2520
        GTTTACCACCCTTTCACTACTATTTAAACGATTTCGACACATAGTCGTTAAACACCTTAA

a           M  V  G  K  *                                             -

        TTATGAAAAGGTTACTGGAACAGACTTAGAGCTTATTCAAATATTAAAAGATCATTATAA
   2521 ---------+---------+---------+---------+---------+---------+ 2580
        AATACTTTTCCAATGACCTTGTCTGAATCTCGAATAAGTTTATAATTTTCTAGTAATATT

a                                                                     -

        TATTTCTTTAGATAATCCCC
   2581 ---------+---------+ 2600
        ATAAAGAAATCTATTAGGGG

a                           -
```
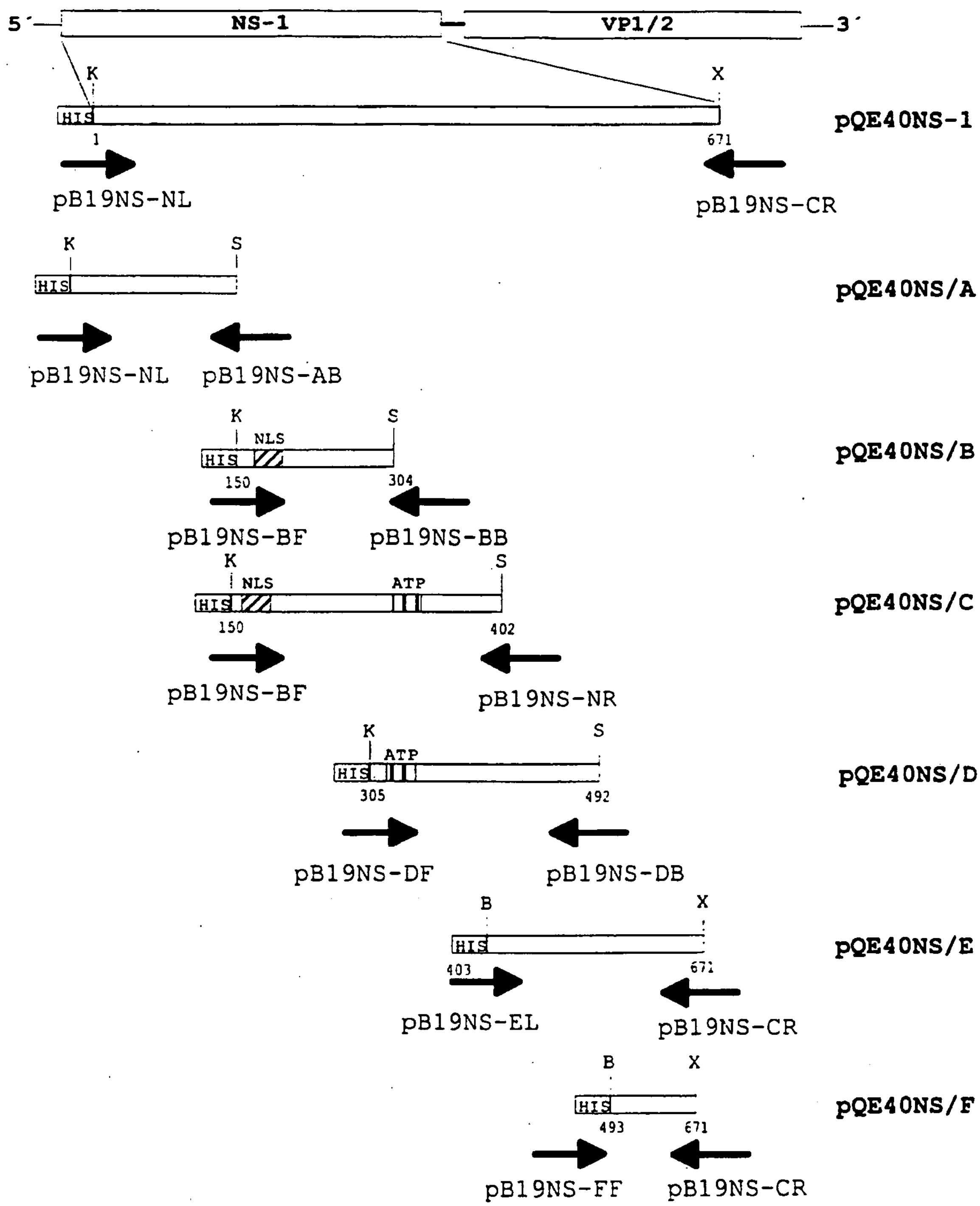

Fig.2: Nucleotide sequence of the primers used for the amplification of different parts of the human parvovirus B19 NS-1 protein.

amplification of	name	sequence 5'to 3'	homology to nt-positions
NS-1	pB19NS-NL	ATTAGGTACCATGGAGCTATTTAGAGGG	435-453
	pB19NS-CR	ATTAGAATTCCTCGAGTTACTCATAATCTACAAAGCT	2431-2451
	pB19NS-1HIS/N	GGAAGATCTATGCATCACCATCACCATCACGAGCTATTTAGAGGGGTG	439-456
NS-A	pB19NS-NL	see above	
	pB19NS-AB	TTATGATCAGTCGACAACATTTAAAGGTATTTTTTTC	864-884
NS-B	pB19NS-BF	TTAGGTACCATGGTATGGTGTGTTACTAATA	886-904
	pB19NS-BB	TTATGATCAGTCGACCAATAGGGGGTCATAG	1315-1330
NS-C	pB19NS-BF	TTAGGTACCATGGTATGGTGTGTTACTAATA	886-904
	pB19NS-NR	ATTAGTCGACTTAGGATCCACGCATTTTTTGATCTAC	1618-1638
NS-D	pB19NS-DF	TTAGGTACCATGGTGGGGCAGCATGTG	1349-1365
	pB19NS-DB	TTAAGATCTGTCGACGGAGGTCTGGGTGGAGGG	1913-1930
NS-E	pB19NS-EL	ATTAGGATCCGTAGCTGTGCCTGGAGTAC	1642-1660
	pB19NS-CR	see above	
NS-F	pB19NS-FF	ATAGGATCCCTCCACCCAGACCTC	1915-1929
	pB19NS-CR	see above	

Fig. 3



5´
NS-1
VP1/2
3´
K
X
HIS
1
671
pQE40NS-1
pB19NS-NL
pB19NS-CR
K
S
HIS
pQE40NS/A
pB19NS-NL
pB19NS-AB
K
S
NLS
HIS
150
304
pQE40NS/B
pB19NS-BF
pB19NS-BB
K
S
NLS
ATP
HIS
150
402
pQE40NS/C
pB19NS-BF
pB19NS-NR
K
S
ATP
HIS
305
492
pQE40NS/D
pB19NS-DF
pB19NS-DB
B
X
HIS
403
671
pQE40NS/E
pB19NS-EL
pB19NS-CR
B
X
HIS
493
671
pQE40NS/F
pB19NS-FF
pB19NS-CR

Fig. 4

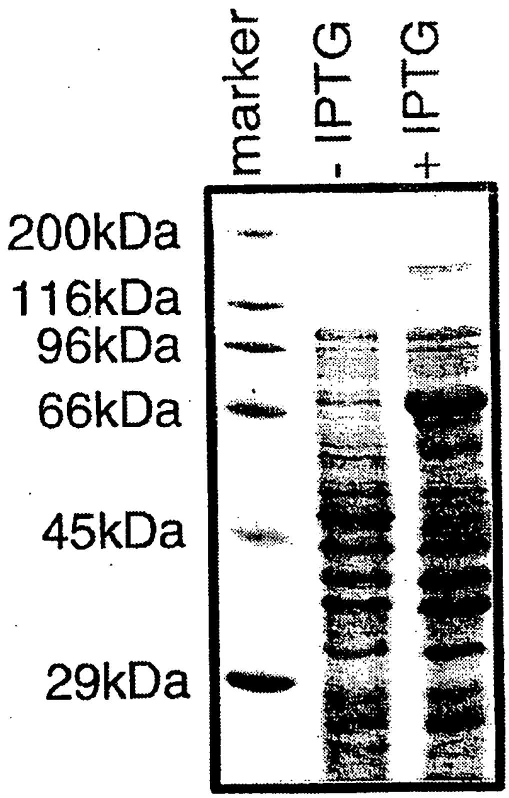


Fig. 5

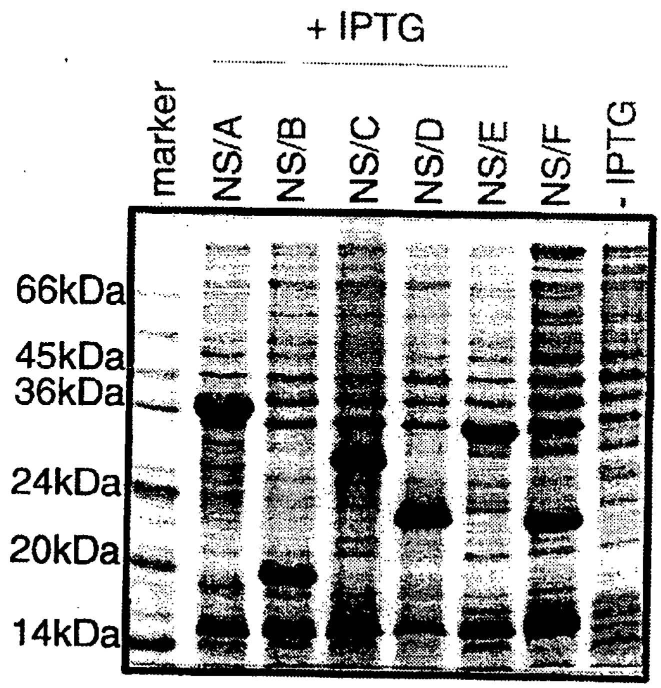

+ IPTG
marker
NS/A
NS/B
NS/C
NS/D
NS/E
NS/F
- IPTG
66kDa
45kDa
36kDa
24kDa
20kDa
14kDa

Fig. 6

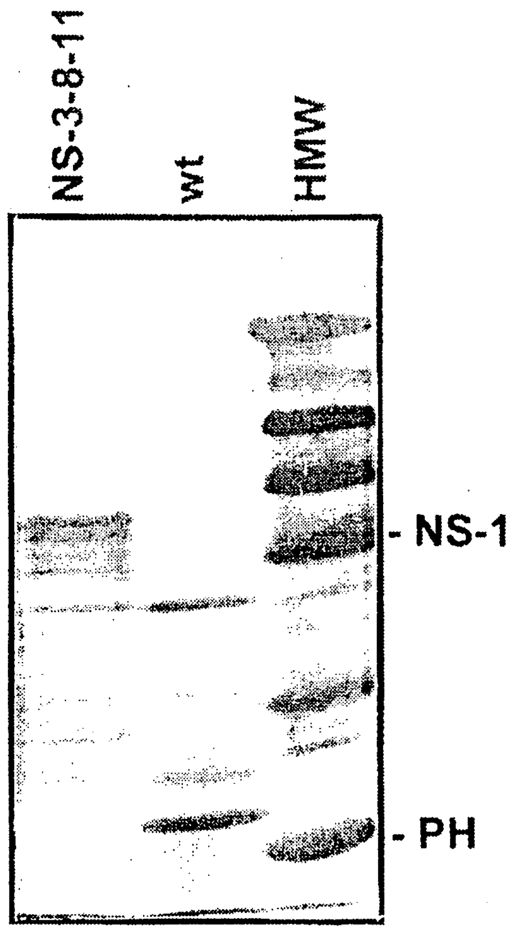


Fig. 7

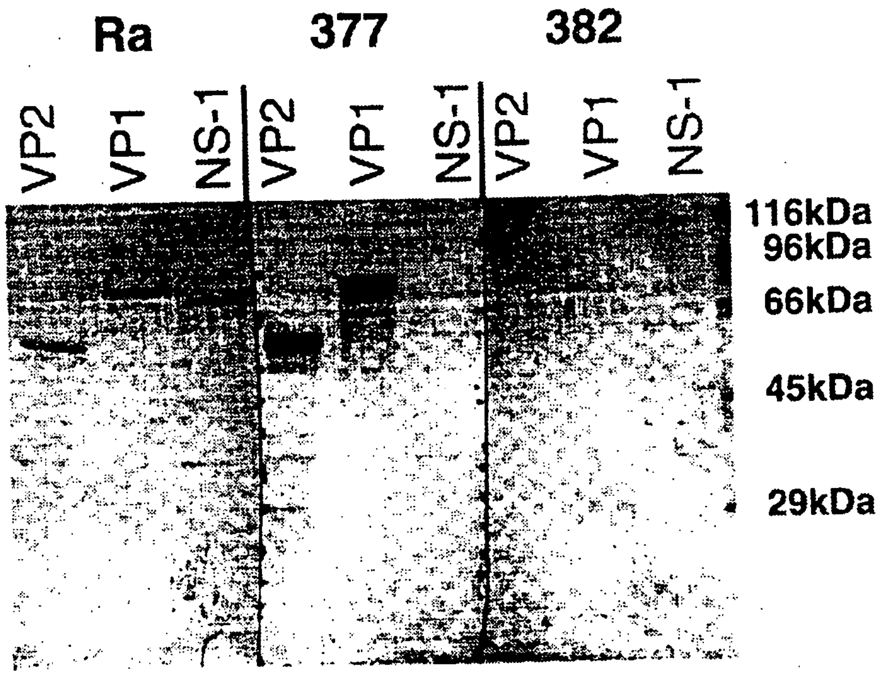
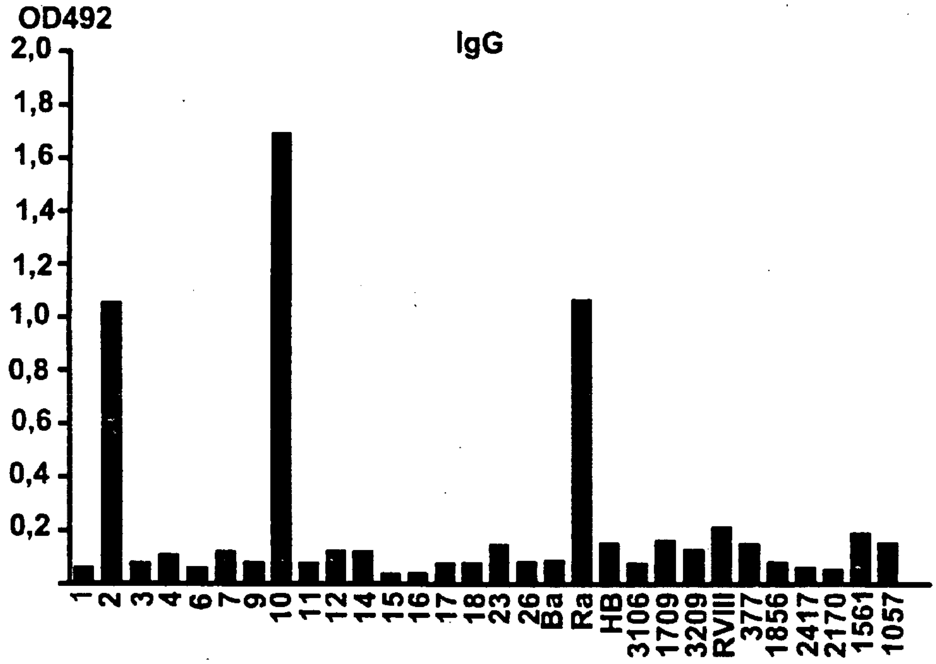

Ra
377
382
VP2
VP1
NS-1
VP2
VP1
NS-1
VP2
VP1
NS-1
116kDa
96kDa
66kDa
45kDa
29kDa

Fig. 8


OD492
IgG
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
1
2
3
4
6
7
9
10
11
12
14
15
16
17
18
23
26
Ba
Ra
HB
3106
1709
3209
RVIII
377
1856
2417
2170
1561
1057

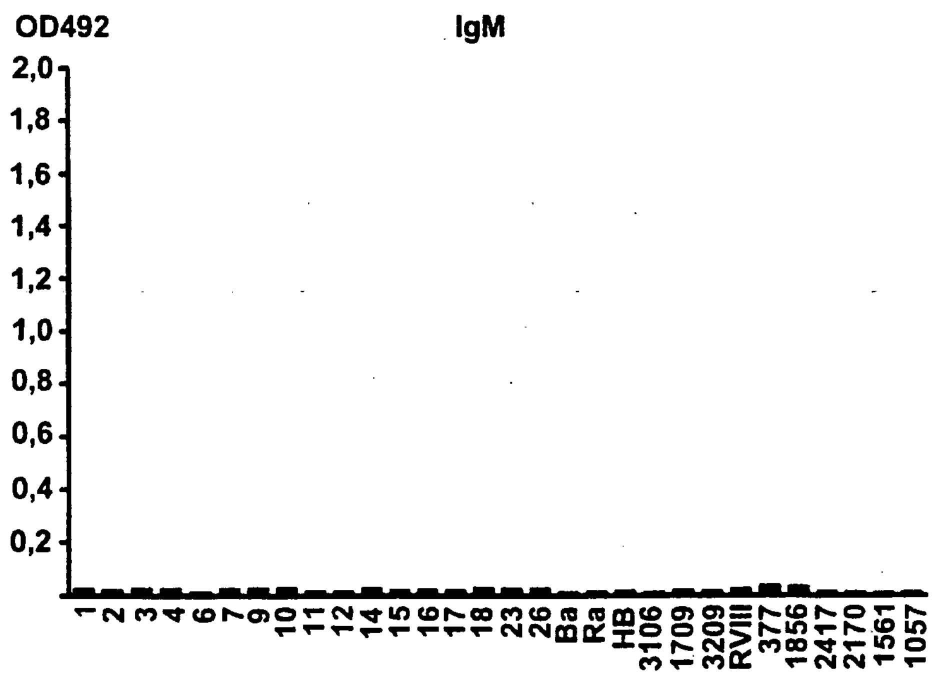

OD492
IgM
2,0
1,8
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
1
2
3
4
6
7
9
10
11
12
14
15
16
17
18
23
26
Ba
Ra
HB
3106
1709
3209
RVIII
377
1856
2417
2170
1561
1057

Fig. 9

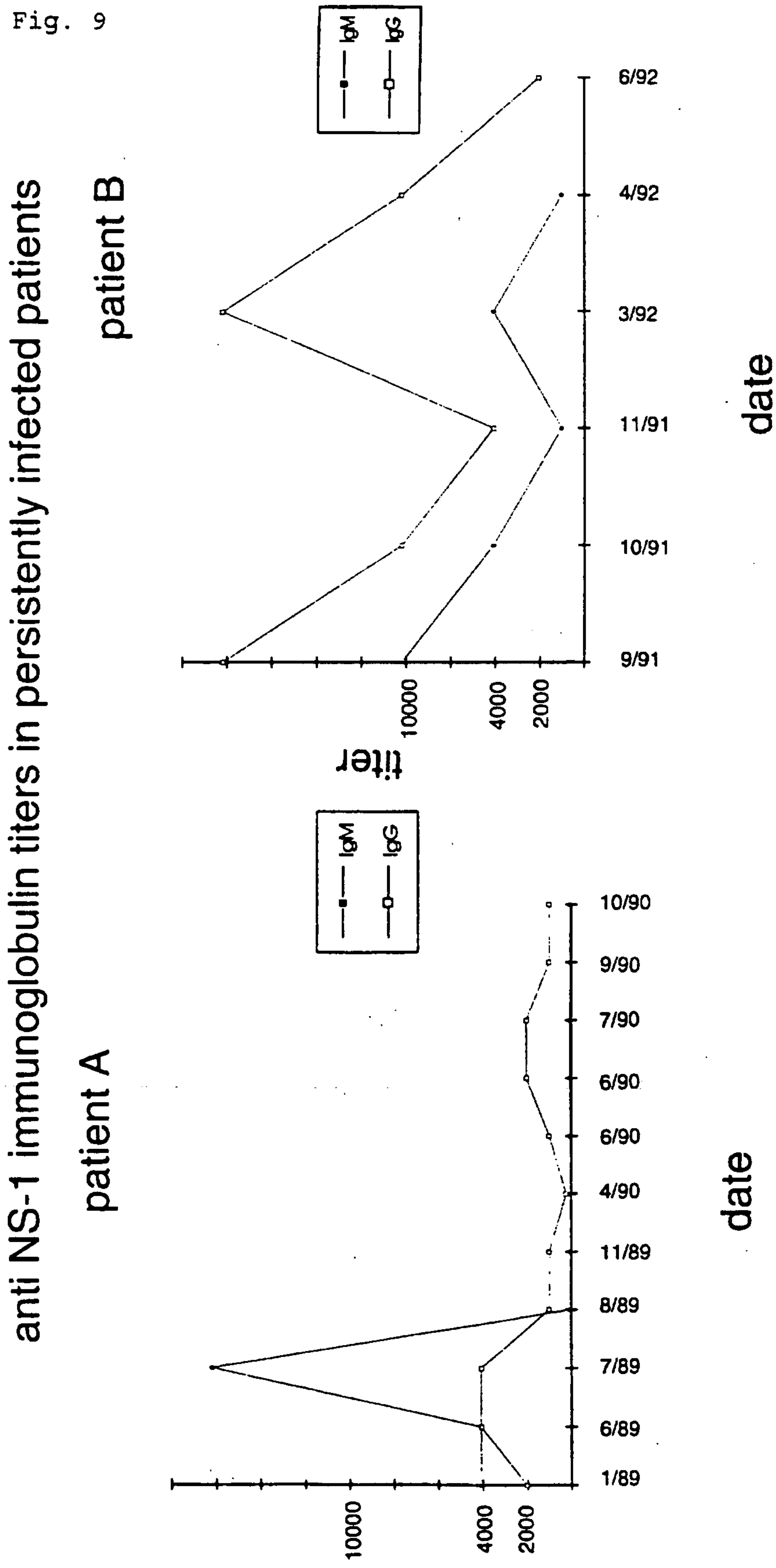
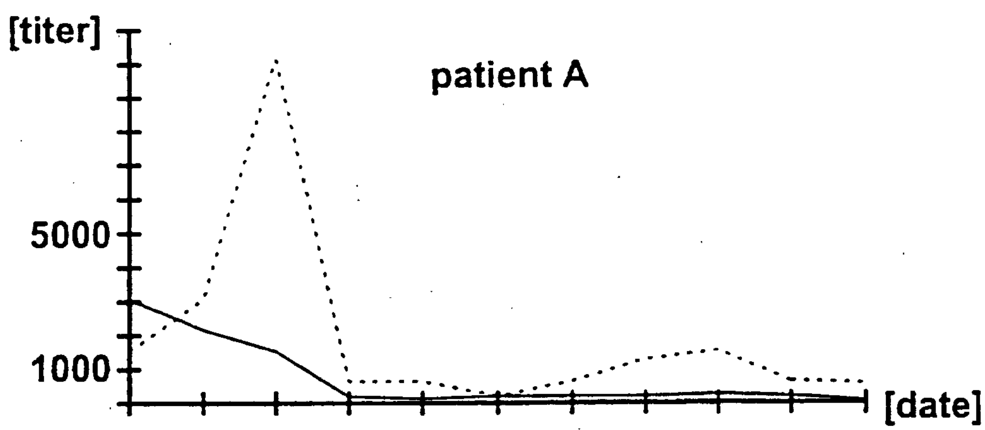

anti NS-1 immunoglobulin titers in persistently infected patients
patient A
patient B
IgM
IgG
titer
10000
4000
2000
date
1/89
6/89
7/89
8/89
11/89
4/90
6/90
6/90
7/90
9/90
10/90
9/91
10/91
11/91
3/92
4/92
6/92

Fig. 10

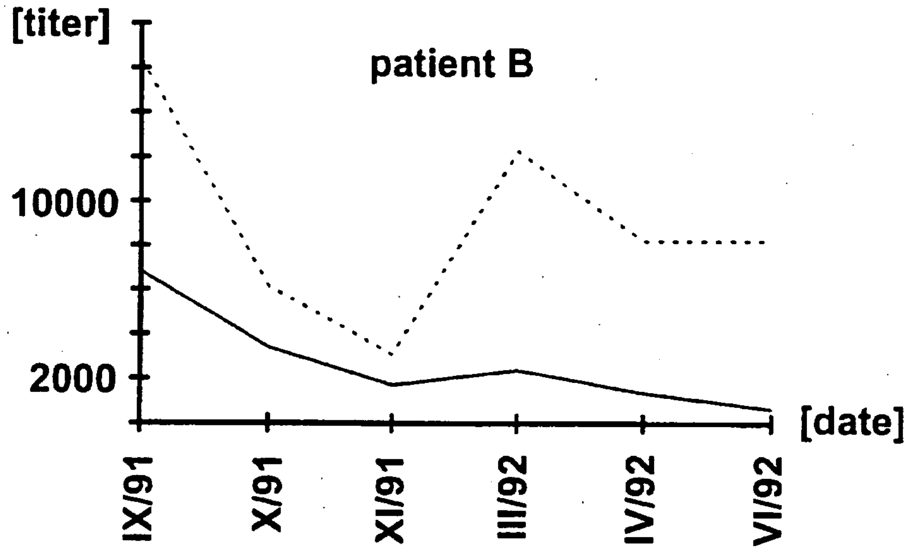

NS/A
NS/B
NS/C
NS/D
NS/E
NS/F
NS-1
DHFR
66kDa
45kDa
36kDa
29kDa
24kDa
20kDa
14kDa

Fig. 11a



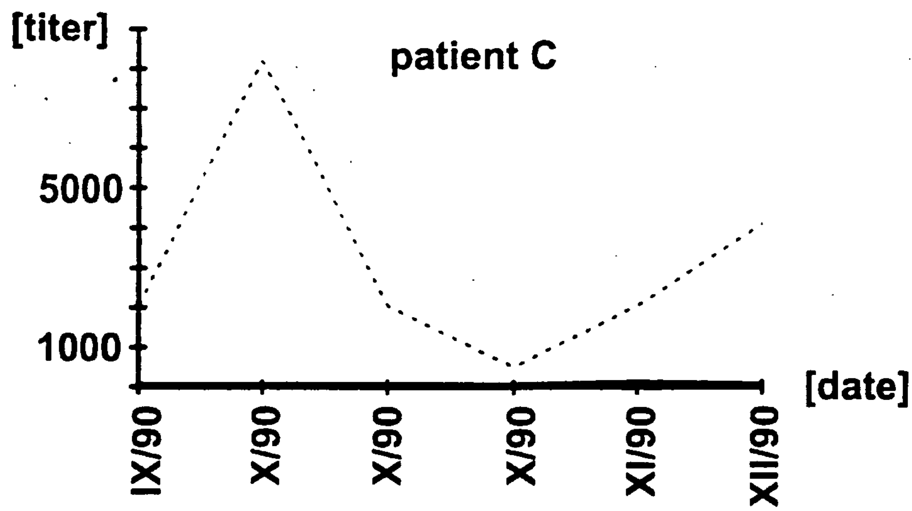
	I/89	VI/89	VII/89	VIII/89	XI/89	IV/90	IV/90	VI/90	VII/90	IX/90	X/90
VP- IgM	+	+	+	+	-	-	+	+	+	-	-
VP-IgG	+	+	-	+	-	-	-	-	+	+	+
B19 DNA(blot)	+	+	+	+	+	+	+	+	+	-	-
PCR	+	+	+	+	+	+	+	+	+	-	-

Fig. 11b



	IX/91	X/91	XI/91	III/92	IV/92	VII/92
VP- IgM	+	+	+	+	+	+
VP-IgG	+	+	+	-	-	+
B19 DNA(blot)	-	+	+	-	-	-
PCR	-	+	+	-	+	-

Fig. 11c



VP- IgM	+	+/-	+/-	+/-	+/-	-
VP-IgG	+	+	+	+	+	+
B19 DNA(blot)	+	+	-	-	-	-
PCR	+	+	+	+	+	+

Note. +: reactive; +/-:near cut-off value; -: not reactive
VP-IgM; VP-IgG: antibodies to capsid proteins
------ : IgG titer; —— : IgM titer

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A3

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(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: VON POBLOTZKI, Andreas [DE/DE]; Maximilian-Aschenauer-Strasse 5, D-93059 Regensburg (DE). MODROW, Susanne [DE/DE]; Ringstrasse 14, D-93177 Altenthann (DE). GIGLER, Andreas [DE/DE]; Königswiesenweg 23, D-93051 Regensburg (DE).

(74) Agent: VOSSIUS & PARTNER; P.O. Box 86 07 67, D-81634 München (DE).

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(54) Title: DNA SEQUENCE AND PROTEIN OF THE NON-STRUCTURAL READING FRAME I OF THE HUMAN PARVOVIRUS B19

(57) Abstract

DNA sequences of the human parvovirus B19 genome coding for the non-structural protein, recombinant DNA molecules containing such sequences, antigens coded within these sDNA sequences of the parvovirus B19 genome coding for the non-structural protein sequences, production of those antigens, generation of antibodies or antibody related molecules directed to such antigens, use of these sequences, antigens, antibodies or antibody related molecules for diagnostic, therapeutic or vaccination purposes.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 95/03758

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/35 C12Q1/68 C07K14/015 C12N1/21 C07K16/08
A61K39/23 A61K39/42 G01N33/569

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N C12Q A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF VIROLOGY, vol. 62, no. 8, August 1988, pages 2884-2889, XP002005736 KEIYA OZAWA ET AL.: "The gene encoding the nonstructural protein of B19 (human) Parvovirus may be lethal in transfected cells" see abstract see page 2886, left-hand column, paragraph 3 - right-hand column, paragraph 1 see page 2887, right-hand column, paragraph 2 - page 2888, right-hand column, paragraph 2 --- -/--	1,3,5-8

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
17 June 1996	12.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Montero Lopez, B

International Application No
PCT/EP 95/03758

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF VIROLOGY, vol. 58, no. 3, June 1986, pages 921-936, XP002005737 ROSEMARY O. SHADE ET AL.: "Nucleotide sequence and genome organization of human Parvovirus B19 isolated from the serum of a child during aplastic crisis " cited in the application see abstract; figure 2 see page 927, left-hand column, paragraph 2 - right-hand column, paragraph 1 see page 930, right-hand column, paragraph 3; figure 7 see page 934, right-hand column, paragraph 3 ---	1
P,X	JOURNAL OF GENERAL VIROLOGY, vol. 76, no. 3, March 1995, READING GB, pages 519-527, XP002005738 ANDREAS VON POBLOTZKI ET AL.: "Antibodies to parvovirus B19 NS-1 protein in infected individuals" see abstract see page 521, right-hand column, paragraph 4 - page 524, left-hand column, paragraph 1 see page 525, left-hand column, paragraph 3 - page 526, left-hand column, paragraph 1 see page 526, left-hand column, last paragraph ---	1-16
P,X	JP,A,07 147 986 (DENKA SEIKEN KK) 13 June 1995 see page 9 - page 10 -----	7,8

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte tional Application No
PC I/EP 95/03758

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP-A-7147986	13-06-95	NONE	